



ORIGINAL RESEARCH PAPER

Obstetrics & Gynaecology

ROUTINE ANALYSIS OF HUMAN SEMEN SAMPLE USING MANUAL SPERM COUNTING AND COMPUTER ASSISTED SPERM ANALYSIS

KEY WORDS:

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ABSTRACT

The semen sample of a 32-year rugby player, healthy and fit and his 29-year-old wife with polycystic ovarian disease, trying to conceive since one year showed normal macroscopic examination and a count of 4.72million/ml by manual counting and 17.2million/ml by CASA. The progressive motility was 10.7% by manual examination and 1% by CASA. The delay in transportation, exposure to high temperature and errors while performing the analysis may lead to asthenozoospermia (decrease in sperm motility). While the other reasons for decreased motility can be due to high fever, severe illnesses, smoking, excessive alcohol, use of recreational drugs, inflammation of the testes and radiation exposure. Hence, a second sperm analysis after treating acute illness, stress, anxiety and proper transportation will be helpful to confirm the diagnosis. Moreover, due to a low count and motility and the female partner suffering from polycystic ovarian disease the couple has to go for Assisted Reproductive Techniques (ART), Intra Cytoplasmic Sperm Injection (ICSI), as the CASA readings show a very low count and motility.

INTRODUCTION

The male reproductive tract consists of a pair of testes (sing. testis) lying in the scrotum attached by a gubernaculum, male sex accessory glands with ducts and the male external genitalia. Each testis is covered with a shiny layer of tunica vaginalis below which is the dense layer of tunica albuginea. The testis is divided into lobules by fibrous septae containing highly coiled seminiferous tubules, which comprise of male germ cells lining the inner wall producing spermatozoa nurtured by Sertoli cells (Figure 1). The interstitial tissue consists of Leydig cells producing testosterone (Jones and Lopez, 2006)

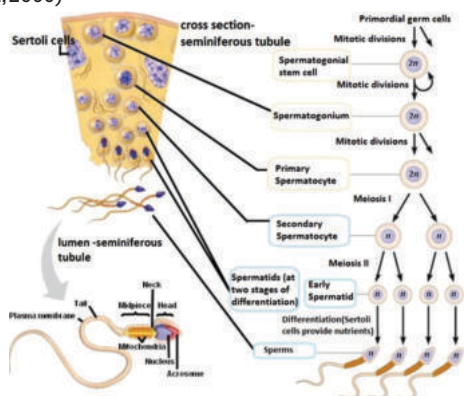


Figure 1- Cross section of the seminiferous tubule- showing various germ cells lining its wall and mature sperms in its lumen. Right side shows the process of spermatogenesis-where primordial germ cells form the primary spermatocyte by mitotic divisions which finally undergoes meiosis to form spermatids. Differentiation takes place in the spermatids to form spermatozoa or sperms (Evans et al., 2013).

During spermatogenesis a diploid spermatogonium transforms into four haploid spermatids. A cross section of seminiferous tubule with the processes of spermatogenesis is explained in Figure 1. The maturation and differentiation of early spermatids into spermatozoa (sperm cells) is called spermiogenesis (Figure 2). In this stage, excess cytoplasm and materials in the cells are digested by Sertoli cells, tails begin to develop, and the DNA is repackaged. In the cytoplasmic space not taken up by the Sertoli cells, mitochondria (used to provide energy for the sperm cells that must swim long distances to get to the female ovum) and an acrosome cap (an organelle with digestive

enzymes) develop. When the cell has undergone these developments, spermiogenesis is complete and the cells are now spermatozoa (sperm cells) and are now mature enough to be released into the lumen of the seminiferous tubule (Evans et al., 2013).

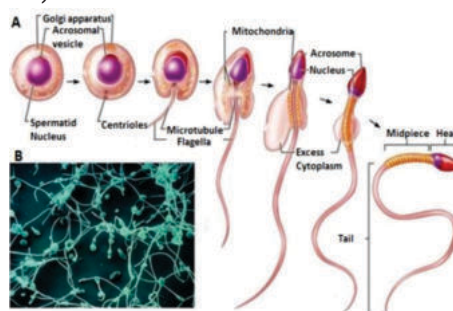


Figure 2- Spermiogenesis. (A)-Maturation and differentiation of the spermatids into spermatozoa (B)-Electron microscope picture of human sperm (Evans et al., 2013).

The spermatozoon or sperm is composed of a head-consisting of the nucleus with tightly packed haploid set of chromosomes, neck- consisting of the centriole, mid piece-containing the mitochondria and tail-with the axoneme (Figure 1 and 2). The acrosome is situated on top of the sperm head and contains enzymes for sperm penetration (Jones and Lopez, 2006).



Figure 3 - Human sperm with 400X magnification under phase contrast microscope. (Available at <http://www.linkam.co.uk/>. Accessed -22/2/2014)

Human sperm under a phase contrast microscope is shown in Figure 3. Semen analysis is the main laboratory investigation for the assessment of male fertility. A semen sample is examined in the laboratory to access several well defined parameters. However, there has been a great difficulty setting these parameters, because some patients with relatively poor semen parameters can still father children(Meniru, 2001). Setting the lower limits is essential to determine infertility in males have extremely low or no sperm in semen. The latest WHO criterion is presented in Table 1.

Table 1- Lower reference limit (5th percentile and their 95% confidence intervals) for semen characteristics (WHO manual of 2010)

Parameter	Lower reference limit
Semen volume(ml)	1.5 (1.4-1.7)
Total sperm number(10 ⁶ per ejaculate)	39 (33-46)
Sperm concentration(10 ⁶ per ejaculate)	15 (12-16)
Total Motility (PR+NP, %)	40 (38-42)
Progressive motility (PR, %)	32 (31-34)
Vitality (Live spermatozoa, %)	58 (55-63)
Sperm Morphology (Normal forms, %)	4.0 (3.0-4.0)

The sperm motility and counts are performed manually or by computer assisted sperm analysis (CASA). The sperm motility is accessed by placing a small volume of undiluted sample on a plain glass slide and covering it with a coverslip. This is followed by counting sperms in specific number of fields and grading them as mentioned in Table 2.

Table 2- Grading of sperms according to their motility

Grades	Motility
A	Rapid progressive motility
B	Slow or sluggish progressive motility
C	Non-progressive motility
D	Immotile

A variety of sperm counting chambers are available for determining the sperm concentration manually. The more commonly used are haemocytometer, Makler counting chamber and the Microcell. An aliquot from a homogenous, mixed semen sample is placed onto a chamber with coverslip at room temperature to distribute it evenly in a very thin layer. Sperm within the grid are counted and calculation is made according to the formula for the type of chamber used(Gardner et al., 2009).

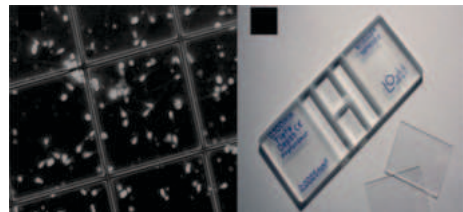


Figure 4- Manual counting of sperm. (A)-Sperms visualized under a 20X microscope on a haemocytometer. (B)Burker haemocytometer (Brightline LO-Laboroptik,Lansing,UK). (Modified from <http://www.uropunkt-hoechststadt.de/>. Accessed- 22/2/2014)

A photomicrograph of human sperms under 20X magnification on Burkler haemocytometer is shown in Figure 4. Sperm count should be performed immediately after loading it onto the chamber to decrease the errors caused by heat from the microscope light (Gardner et al., 2009).

CASA was initially developed to improve the accuracy of manual semen analysis. Its goal is to establish a standardised, objective and reproducible test for sperm concentration, motility and morphology. The automated sperm movement measurements known as sperm kinematics include straight line velocity, curvilinear velocity and mean angular

displacement.

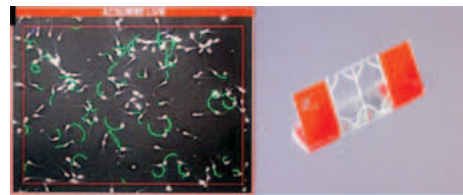


Figure 5- CASA. (A)-Leja slide (Gynotec Malden, Nieuw-Vennep, The Netherlands) -30µl with two chambers (B)-Sperms visualised under CASA systemshowing velocity distribution (Modified from <http://www.equine-reproduction.com/> Accessed-22/2/2014)

CASA is performed by placing sperm on a specialized slide – Leja slide (Figure 5), Makler or Microcell chamber and digitalised image pixels with changing locations are recorded frame by frame. The changing locations of each sperm are recorded and their trajectories computed. Persistent questions about the validity and reproducibility of results have kept CASA from becoming a standard procedure (Gardner et al., 2009). CASA has been consistently shown to outperform manual methods in terms of accuracy as well as in the variety of parameters obtainable to describe sperm motility; however, its high cost has hindered widespread adoption. Many reports in the literature suggest that CASA systems cannot provide accurate values as compared to well-controlled manual analyses(Ginsburg et al., 1998); (Mahony et al., 1998). In addition, that much of the information necessary to reproduce data obtained with commercial systems is proprietary information, and thus, not available. Consequently, this limits the ability to conduct comparative evaluations(Wilson- Leedy & Ingermann, 2006).

Male Factor Infertility

A male factor is solely responsible in about 20% of infertile couples and contributory in another 30-40% (Thonneau et al., 1991). If a male infertility factor is present, it is almost always found out by the finding of an abnormal semen analysis, although other male factors may play a role even when the semen analysis is normal. Any process that can affect sperm production and quality is potentially harmful to male fertility. The major causes of male infertility include varicocele, genital tract obstruction, testicular failure, cryptorchidism, idiopathic, gonadotoxin exposure, genetic conditions, infections, hormonal dysfunction, immunological conditions, ejaculatory/sexual dysfunction, cancer and systemic diseases (Esteves et al., 2011). The nomenclature related to semen quality is mentioned in Table 3.

The purpose of the male evaluation is to know these conditions when present. Identification and treatment of reversible conditions may improve the male's fertility and allow for conception through intercourse. Thus, an appropriate male evaluation may allow the couple to understand the basis of their infertility and to obtain genetic counselling when appropriate. If specific corrective treatment is not available, it still may be possible to use assisted reproductive techniques such as testicular or epididymal sperm retrieval with intracytoplasmic sperm injection (ICSI). Or else, such couples may consider therapeutic donor insemination or adoption. Finally, male infertility may sometimes be a presenting manifestation of an underlying life-threatening condition(Honig et al., 1994).

The aim of this practical is to carry out laboratory tests performed on human semen samples and to analyse human spermatozoa by manual sperm motility assessment and counting and CASA along with studying different sperm parameters like motility, velocity distribution and kinematics.

Table 3- Nomenclature related to semen quality, WHO manual,2010

aspermia	no semen (no or retrograde ejaculation)
asthenozoospermia	Percentage of progressively motile (PR) spermatozoa below the lower reference limit
asthenoteratozoospermia	percentages of both progressively motile (PR) and morphologically normal spermatozoa below the lower reference limits
Azoospermia	No spermatozoa in the ejaculate (given as the limit of quantification for the assessment method employed)
cryptozoospermia	spermatozoa absent from fresh preparations but observed in a centrifuged pellet
haemospermia (haematospermia)	Presence of erythrocytes in the ejaculate
Leukospermia (leukocytospermia, pyospermia)	presence of WBCs in the ejaculate above the threshold value
Necrozoospermia	Low percentage of live, and high percentage of amotile, spermatozoa in the ejaculate
Normozoospermia	total number (or concentration, depending on outcome reported)* Of spermatozoa, and percentages of progressively motile (PR) and morphologically normal spermatozoa, equal to or above the lower reference limits
Oligoasthenozoospermia	total number of spermatozoa, and percentage of progressively motile (PR) spermatozoa, below lower reference limits
oligoasthenoteratozoospermia	total number (or concentration, depending on outcome reported)* of spermatozoa, and percentages of both progressively motile (PR) and morphologically normal spermatozoa, below the lower reference
Oligoteratozoospermia	Total number (or concentration, depending on outcome reported)* of spermatozoa, and percentage of morphologically normal spermatozoa, below the lower reference limits
Oligozoospermia	total number (or concentration, depending on outcome reported)* of spermatozoa below the lower reference limit
teratozoospermia	Percentage of morphologically normal spermatozoa below the lower reference limit

*Preference should always be given to total number, as this parameter takes precedence over concentration.

MATERIAL AND METHODS

The semen samples used in this practical were provided by the Oxford Fertility Unit (OFU), Oxford business park north, Oxford, UK, after a written, valid and informed consent from the patients. Post liquefaction and various tests carried out in the OFU the samples that were surplus to requirement were given along with their respective case histories.

Basic semen tests carried on the samples were its appearance, volume, viscosity, pH and microscopic examination for motility, morphology and concentration. The appearance of semen was noted. A normal semen sample has a homogenous grey- opalescent appearance. If sperm concentration is low, it appears less opaque. The volume was measured by aspirating the whole sample in to a 5ml or 10ml pipette.

Sample viscosity was estimated by gentle aspiration into a 5ml pipette and then allowing the semen to drop by gravity and observing the length of thread produced. The sample is said to have a normal viscosity if it leaves the pipette as small discrete drops, reduced viscosity if it is watery, increased viscosity if it forms a thread while leaving the pipette and greatly increased if the sample does not pass the pipette.

The pH of sample was measured by spreading a small amount of sample on a pH strip (range-6.5-10.0) evenly. The colour of the strip was then compared to the calibration strip and the pH recorded.

The microscopic investigation was done by preparing a 'wet prep' slide, 10 µl of semen sample was placed on a clean glass slide and covered with a coverslip. This slide was examined microscopically for motility, morphology and general appearance. Motility was measured by grading the sperm scanned systematically under the 20X objective of microscope as shown in Table 2. A specialized hand counter was used for counting and atleast 200 sperm and 5 fields of view were counted to achieve a low counting error. These values were then expressed as percentages. The process was repeated on a second drop, and the values averaged, provided they are within 95% confidence limits (the largest

value in the first drop has a difference of less than 10 from the corresponding value in the second drop). If the values were not in the 95% confidence limits, a third drop was accessed. These confidence limits applied only when 200 sperm were counted. The slides were also scanned for complete liquefaction of the sample and presence of cellular elements other than sperm (round cells, debris, erythrocytes, bacteria and protozoa) and agglutination of sperm.

Manual sperm counting for each sample was done using a standard Burkcr haemocytometer (Bright line,LO-Laboroptik, Lansing, UK). 10µl of semen sample was diluted with 190µl of water (dilution 1:20), mixed on a vortex mixer and 10µl of the diluted sample was placed on each grid. The cells were counted on 200X magnification on the grid under negative phase contrastin the red square (Figure 6). If the number of sperm were less than 200 than a second square was counted and repeated for the other half of the haemocytometer. The total sperm concentration was calculated using the following formula.

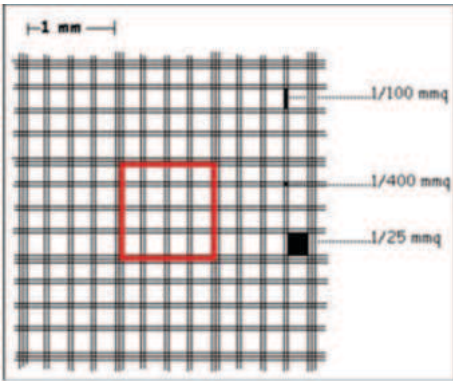


Figure 6- Grid on the Burkcr haemocytometer for the counting of sperm. The numbers of sperm heads falling in the red square were counted. Modified from <http://www.selfpediatrico.it/> .Accessed-22/2/2014)

Sperm concentration in 1 ml= (Number of sperm in all counted large squares÷ number of squares) x 10,000 x dilution factor

To express sperm concentration in millions /ml, the resulting value is divided by 10⁶.

The CASA (Hamilton Throne, Danvers, MA, USA) analysis was performed by transferring appropriate amount of sperm (5µl) into one chamber of a 20µm sized Leja slide (Gynotec Malden, Nieuw-Vennep, The Netherlands) and placed into a slide warmer of the microscopic stage to acclimatise for 2 minutes with a pipette tip (Figure 5). The Hamilton Thorne computer assisted sperm analysis system was set up to analyse the human sperm (Table 4).

Table 4- Program set up for CASA

Program set up	Standard (human)(set up 1)
Leja slide	20µm
Objective lens	x 10 negative phase
Condenser setting	Ph1
Volume of sperm on Leja slide	5µl (neat, undiluted)
Total volume of sample	0.6ml

The samples were analysed counting more than 600 cells in total from atleast five different fields of view. The results from the data were added in one central data bank and printed, followed by statistical analysis.

RESULTS

The semen sample was of a 32-year-old rugby player, fit and

healthy with a 29-year-old wife having polycystic ovarian syndrome (PCOS) and trying to conceive since 1 year. The macroscopic semen analysis showed the following results (Table 5).

Table 5-Results of macroscopic semen analysis

Macroscopic examination	Results
Appearance	Normal
Volume	0.6ml
Viscosity	Normal
pH	8.5

The microscopic examination of sperm for motility was carried according to the grading of motile and immotile sperms. The values for the provided sample are listed in Table 6.

Table 6- Motility of sperm in semen sample provided

Grade	A	B	C	D	Total
Slide 1	12	12	37	168	229
Slide 2	14	10	40	151	215
Percentage	5.8%	4.9%	17.3%	72%	444 (100%)

Total number of sperms with good motility = (A+B) = 10.7% Morphological features of semen sample provided are listed in Table 7.

Table 7- Morphological features of the provided semen sample

Morphological features	Results
Round Cells	<5 million/ml
Agglutination	None
Bacteria/ debris	None

The results of manual sperm counting to determine the concentration of sperm in the semen sample by the use of Burker haemocytometer is shown below.

Sperm concentration in 1 ml= (Number of sperm in all counted large squares÷ number of squares) x 10,000 x dilution factor.

Sperm concentration in 1 ml= (226+246/2) x 10000 x 20 = 4.72 x 10⁷ sperm/ml Therefore, total sperm concentration= 47.2 million/ml.

CASA Analysis

The total sperm count with CASA was 17.9 million/ml and total number of motile sperms is

0.5 million /ml with a progressive motility of 1%. The comparison of the results obtained by manual sperm counting and CASA are graphically represented in Figure7.

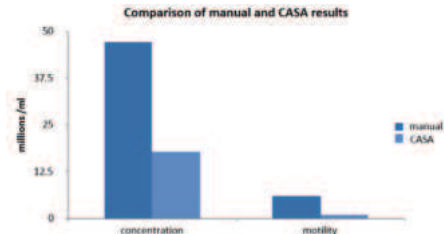


Figure 7- Graphical chart comparing manual and CASA results for different parameters in semen analysis.

Data obtained by CASA of the sperm kinetics and motility from the sample is tabulated in

Table 8. Appendix 2 shows the definitions of the various parameters.

Table 8- Data showing sperm kinetics of human sperm analysed by CASA

Sperm Kinematics	
Parameter	Human sperm
VAP (Average Path velocity) (µm/s)	40.2 ± 10.4
VSL (Straight line velocity) (µm/s)	34.7 ± 9.3
VCL (Curvilinear velocity) (µm/s)	56.6 ± 10.9
ALH (Amplitude of lateral head displacement) (µm)	2.5 ± 1.2
BCF (Beat cross frequency) (Hz)	16.8 ± 17
STR (Straightness) (%)	87 ± 5
LIN (Linearity) (%)	61 ± 12
Elongation (%)	69 ± 15
Area (µmsq)	14.9 ± 1.1

DISCUSSION

The aim of this practical was to carry out laboratory tests performed on human semen samples and to analyse human spermatozoa by manual sperm motility assessment and counting and CASA along with studying different sperm parameters like motility, velocity distribution and kinematics.

Semen analysis, chiefly sperm counting, an importanttest performed in all infertility laboratories sometimes show wide variations in the same sample.Moreover, this variability is expectedto adversely affect patient care when results for semen analysis parameters (like sperm concentration, motility, and vitality) on thesame semen sample are reported as normal by one laboratory andas consistent with infertility by another laboratory(Auger et al., 2000). Therefore, there have been calls for global standardization of this test(Cooper et al., 2002). In this practical, the comparison between manual and CASA results for sperm counting showeda differenceof 34.6% (Figure 7) suggesting a need for standardization of these tests. This proves the subjective sperm quality evaluation while working with male gametes, depends onthe skill, perception, and training of the researcher whoevaluates the sperm samples (Kime et al., 2001)(Rurangwa et al., 2004).

In United Kingdom, NEQAS (National External Quality Assessment Service), an independent non-profit organisation, provides external quality assessment for clinical laboratories that carry out examinations in general bacteriology, virology, serological testing, blood donor testing (blood borne viruses and syphilis) and parasitology. Hence, there is quality assessment of embryologists regularly. The UK NEQAS facilitates optimal patient care by providing a comprehensive external quality assessment in laboratory medicine. Through education and promotion of best practice, this organization helps ensure the results of clinical laboratory investigations are reliable and comparable, wherever they are produced throughout the UK. The NEQAS movies are recorded at the Manchester Andrology Laboratory and are regularly sent to UK fertility units to monitor semen analysis standards as a part of the UK National External Quality Assessment Service (<http://www.ukneqas.org.uk/>). Embryologists routinely assess the digital movies in the same way that they would during clinical practice. The data is returned to NEQAS for analyses and comparison to other UK fertility units.

The provided sample had a sperm count of 47.2million/ml by manual counting and 17.2million/ml by CASA and progressive motility was 10.7% by manual examination and 1% by CASA, which was low as per the WHO criteria. Hence, a repeat semen analysis is generally recommended.Results from at least two, but preferably three, separate seminal analyses must beobtained before a definitive conclusion can be drawn, aswide biological variability may exist within the very same individual. The interval between the analyses is

arbitrary and is generally recommended to be 1-2 weeks. Ejaculatory abstinence should occur for a minimum of two days to a maximum of seven days, with two to three days being ideal (Esteves et al., 2011) (WHO manual, 2010).

The main causes of asthenozoospermia are ultrastructural defects in the sperm flagellum including genetically inherited and congenital defects, degeneration due to genital infections, oxidative stress, cryopreservation and antisperm antibodies (Lee et al., 2009). Exposure to heat, varicocele, excessive consumption of alcohol and smoking may also be responsible for altered sperm motility (Gardner et al., 2009) (Pant, 2013).

Only a small proportion of subfertile males are amenable to treatment. Fortunately, high female fecundity can often compensate for presence of low sperm concentrations (Collins et al., 1995). If fewer than 1-2 million motile sperms are present after sperm preparation, IVF is normally indicated. The results of IVF in the treatment of male factor infertility are determined primarily by age of the woman (Forti and Krausz, 1998), the degree of sperm motility and sperm morphology (Auger et al., 2000) (Repping et al., 2002) (Gardner et al., 2009). Many studies have reported a strong correlation between impaired semen parameters and fertilization capacity in IVF, and when severe male factor is present total fertilization failure may occur (Gardner et al., 2009). In many centres a post wash total motile sperm count of less than 500 000 is considered to indicate ICSI treatment (Devroey et al., 1998) while others apply a cut off value of 1 million (Gardner et al., 2009). These values remain largely arbitrary, since few reliable data is available for the prediction of chance of total fertilization failure (Repping et al., 2002).

Although ICSI has transformed the fertility prognosis for couples with severe male factor infertility, the appropriate indications of ICSI remain controversial (Devroey and Van Steirthege, 2004). While in some countries ICSI tends to be restricted to treating severe oligoasthenospermia and total fertilization failure, other European and US centres apply a more liberal policy to the use of ICSI. However, absolute indications for ICS are agreed to include the use of microsurgical (epididymal or testicular) spermatozoa (Gardner et al., 2009)

ICSI with ejaculated spermatozoa can be used successfully in patients with fertilization failures after conventional IVF or other assisted fertilization procedures, and also in patients who cannot be accepted for these procedures because too few morphologically normal and progressively motile spermatozoa are present in the ejaculate (De Vos, 2000). In cases of severe asthenozoospermia ICSI is used and the results are much better when motile sperm can be selected (de Mendoza et al., 2000) (Esteves et al., 2011).

Therefore, in this couple where the semen analysis of a 32 year old male is suggestive of an oligoasthenozoospermia (with differences in the manual analysis and CASA) a repeat semen analysis test is necessary along with a complete history and physical examination to rule out medical disorders and additions. Moreover, considering the female partner is suffering from PCOS, use of ICSI than conventional IVF seems the treatment of choice.

Appendix 1
 Setting up the CASA system

Steps	Procedure
1-info	Select the <i>info</i> menu and input total amount of original sperm sample. Occasionally, it may be necessary to dilute the sperm samples. If diluted input the dilution factor
2-set-up	Using the microscope light control, ensure that the photometer reading is around 50 and focus on the sperm. Get a good clear image of moving sperm at this point of time
3-acquire	Select <i>acquire</i> menu followed by <i>scan</i>
4-results	Click <i>results</i> to see the first page
5-add scan	Check the sperm cells counted, move the field and click <i>add scan</i>
6 - Count cells	Ensure that the counted cells are > 800 sperm cells, if not add scans till the number is reached
7-Playback	Print the results and <i>playback</i> the last field of view that will highlight the motility groupings.

Appendix 2
 Typical parameters obtained from CASA (Gardner, et al., 2009)

Symbol	Name	Definition
VSL	Straight-line velocity	Average velocity of sperm head along a straight line from its first position to last position
VCL	Curvilinear velocity	Average velocity of sperm head along its actual trajectory
VAP	Average path velocity	Average velocity of sperm head along its average trajectory
LIN	Linearity	Linearity of curvilinear trajectory (VSL/VCL)
WOB	Wobble	Degree of oscillation of actual sperm head trajectory around its average path (VAP/VCL)
STR	Straightness	Straightness of actual path (VSL/VAP)
ALH	Amplitude of lateral head displacement	Amplitude of variations of actual sperm head trajectory about its average trajectory
BCF	Beat cross frequency	Average rate at which the actual sperm trajectory crosses the average path trajectory

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